

Discrimination of the Hoechst Side Population in Mouse Bone Marrow With Violet and Near-Ultraviolet Laser Diodes

William G. Telford^{1*} and Ella G. Frolova²

¹Experimental Transplantation and Immunology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

²Laboratory of Mammalian Genes and Development, National Institute of Child Health and Development, National Institutes of Health, Bethesda, Maryland

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Background: Discrimination of stem cells with flow cytometric analysis of Hoechst 33342 efflux by the ABCG2 transporter (termed the Hoechst *side population*, or SP technique) is a valuable methodology for identifying bone marrow progenitors enriched with stem cells. Unfortunately, it requires a ultraviolet (UV) laser source, usually necessitating an expensive and maintenance-intensive argon- or krypton-ion gas laser on a large-scale cell sorter. In this study, we evaluated the ability of recently available violet and near-UV laser diodes to discriminate Hoechst SP on smaller cuvette-based flow cytometers.

Methods: Violet laser diodes (emitting at 408 and 401 nm) and a near-UV laser diode (emitting at 370 nm) were mounted on a BD Biosciences LSR II and evaluated for their ability to discriminate Hoechst SP in murine bone marrow.

Results: The violet laser diodes discriminated the Hoechst SP, but with poorer resolution than with the standard UV gas laser on a large-scale cell sorter. The near-UV laser diode, in contrast, gave excellent Hoechst SP resolution.

Conclusions: These evaluations indicated that near-UV laser diodes give excellent Hoechst SP resolution on cuvette-based instruments. As the next generation of cell sorters integrate cuvette-based cell interrogation into conventional jet-in-air cell separation, these laser sources should become applicable for analysis and physical separation of Hoechst SP cells. Cytometry Part A 57A:45–52, 2004. © 2003 Wiley-Liss, Inc.

Key terms: stem cell; Hoechst 33342; side population; near-ultraviolet laser diode

Hematopoietic stem cells are objects of considerable interest in the development and reconstitution of the mammalian immune system. Considerable attention is currently directed at identifying the phenotype of these presumably totipotent progenitors. An important recent development in this area is the identification of the Hoechst *side population* (SP) (1). When unpurified murine bone marrow cells are labeled with the membrane-permeant DNA binding dye Hoechst 33342, a very small fraction of cells extrudes this dye via a membrane pump (1–3). Analysis of these cells on a flow cytometer equipped with an ultraviolet (UV) laser source permits detection of these cells; when Hoechst-labeled cells are analyzed simultaneously through blue and red emission filters, the SP forms a dim tail extending from the normal G1 cell populations. These cells can reconstitute the bone marrow of lethally irradiated mice at an ED₅₀ of fewer than 100 cells, indicating that they are highly enriched for totipotent stem cells (1). The SP cell subpopulation is also enriched for cells expressing the murine stem cell markers Sca-1 and c-kit, further suggesting that they contain very early

hematopoietic progenitors. Recent studies with stable transfectants and knockouts have demonstrated that the causative pump is breast cancer resistance protein 1, or ABCG2 transporter, a member of the ABC cassette membrane protein family; transfection of cells with the ABCG2 gene results in an SP-like phenotype, and ABCG2 transgenic mice have no SP phenotype in their stem cells (4–7). Interestingly, the bone marrow and peripheral immune system in these knockouts is normal, suggesting that the ability to efflux Hoechst 33342 is characteristic of stem cells but not necessary for function (7). Similar SP subpopulations have been observed in primates and humans (8,9). The SP phenotype therefore has become an important marker for stem cell activity in the identifica-

*Correspondence to: William G. Telford, Ph.D., National Cancer Institute, Building 10 Room 12C121, 9000 Rockville Pike, Bethesda, MD 20892.

E-mail: telfordw@mail.nih.gov

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tion of these cells and in their physical separation by fluorescence-activated cell sorting.

There are, however, a number of practical difficulties in the technology surrounding detection of SP cells. Hoechst 33342 requires UV excitation, a wavelength that is comparatively difficult to achieve from a laser source. The most common sources of UV laser light are water-cooled argon- or krypton-ion gas lasers (providing excitation from 337 to 367 nm); these lasers are large, expensive to purchase and operate, and very maintenance intensive. Their large size has limited their use to large flow cytometers and cell sorters, such as the BD Biosciences FACS Vantage (Becton Dickinson, San Jose, CA), the Cytomation MoFlo, or the Beckman Coulter Altra (Beckman Coulter, Hialeah, FL). Because these instruments are designed to physically sort cells by electrostatic deflection, their sample streams are not confined to quartz cuvettes but are jet-in-air; as a result, their light collection optics are inherently inefficient. This optical inefficiency in turn results in a requirement for high laser power levels to excite any cell-associated fluorescent probes. The result of all of these limitations is the need for a large-scale cell sorter equipped with a large, powerful UV laser to carry out Hoechst SP analysis of stem cells. This presents a serious limitation to the application of SP analysis in stem cell biology, because few investigators can afford to purchase or maintain the equipment required to carry out this relatively simple technique. There is a real need to be able to carry out Hoechst SP analysis on smaller, less expensive flow cytometers.

At the core of this problem is the ability to generate near-UV or UV laser light from sources other than gas lasers. Laser diodes are a recent and important development in this area; although these lasers initially were limited to the infrared and red emission range (10), recent developments in gallium nitride diodes have resulted in blue, violet, and near-UV laser diodes capable of emission levels appropriate for flow cytometry (11–15). Violet laser diodes have been found useful for exciting several fluorescent probes relevant to cell biology (such as the DNA dye 4,6-diamidino-2-phenylindole, the phenotyping probes Cascade and Pacific Blue, and the expressible protein cyan fluorescence protein) and are already being installed in several commercial flow cytometers (12–15). Near-UV laser diodes at power levels appropriate for flow cytometry (5–20 mW) are currently in the prototype stage but should be commercially available at the time of publication (15). If these laser sources are able to excite Hoechst-labeled cells and discriminate SP, it would make this important technique far more accessible to a wider variety of flow cytometers and investigators; laser diodes can be incorporated into a far wider group of flow cytometers than large gas sources (15). In this study, we therefore evaluated several laser diode sources for their ability to discriminate SP, ranging in wavelength from the violet to the near-UV (408–370 nm). Because these lasers possess relatively low power levels relative to traditional gas sources, we evaluated them on a cuvette-based flow cytometer (BD Biosciences LSR II).

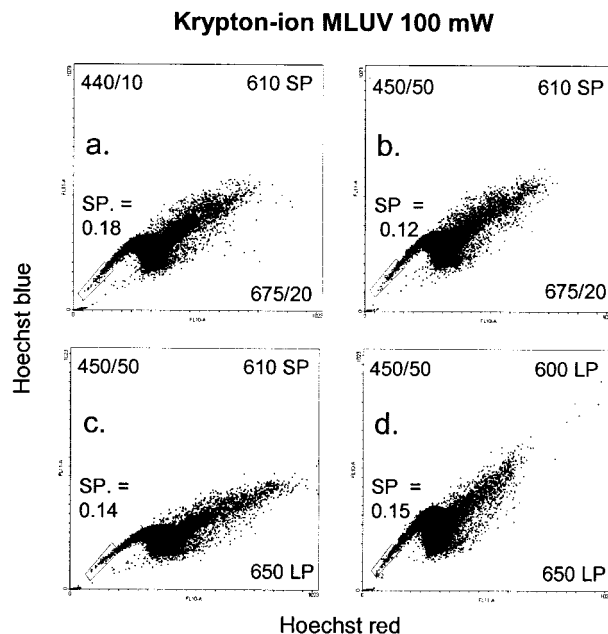


FIG. 1. **a–d**: Hoechst SP analysis of unpurified mouse bone marrow on a FACS Vantage DiVa cell sorter equipped with a krypton-ion laser emitting at an MLUV of 100 mW, using the indicated filter combinations. The Hoechst blue filter is indicated in the upper left corner of each cytogram, the Hoechst red filter in the lower right corner, and the dichroic in the upper right corner. SP gates were drawn by using the funitremorgin C inhibitor samples (not shown) to set cutoffs.

MATERIALS AND METHODS

Mice and Cells

BALB/c female mice 4 to 12 weeks of age (Jackson Laboratory, Bar Harbor, ME) were maintained in the National Institutes of Health (NIH), National Institute of Child Health and Development single pathogen-free barrier animal colony and killed immediately before bone marrow aspiration according to NIH guidelines. Bone marrow was extracted by fine-needle aspiration and washed twice with Hank's balanced saline solution (HBSS) containing 2% fetal bovine serum (FBS) and 10 mM HEPES. Some bone marrow samples were initially incubated with anti-Fcγ2/Fcγ3 antibody for 10 min at 4°C, followed by incubation with phycoerythrin-conjugated antibodies against the lineage markers B220, Ter-119, CD3, Gr-1 and Mac-1 (BD Pharmingen, San Diego, CA) for 20 min at 4°C. Cells were washed and subsequently incubated with anti-phycoerythrin antibody-conjugated paramagnetic beads (Miltenyi Biotec, Auburn, CA) for 20 min. Lineage-positive cells were then removed with an AutoMACS cell separation unit (Miltenyi Biotec) using the normal depletion program. The lineage-positive (SP-depleted) and lineage-negative (SP-enriched) populations were washed in the above buffer and counted. A549 lung carcinoma cells were obtained from the American Red Cross (Rockville, MD) and passaged in Dulbecco's minimum essential medium containing 10% FBS. These cells were removed from their growth substrate with trypsin and ethylene-diamine-

Krypton-ion MLUV 100 mW

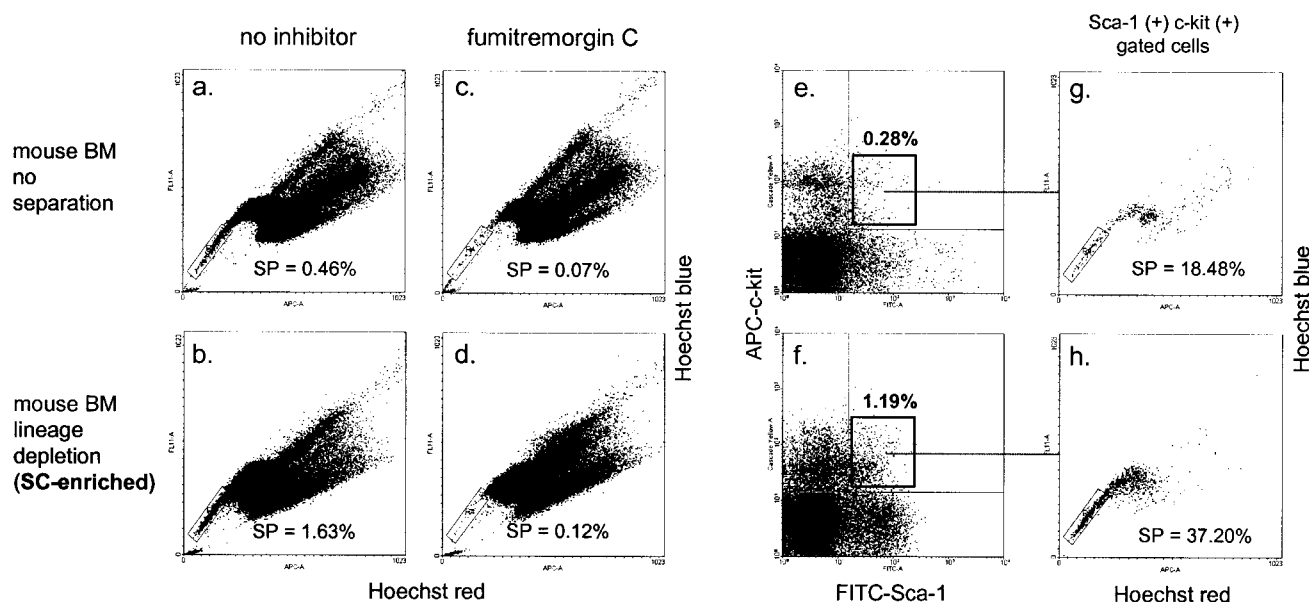


FIG. 2. Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted (b,d,f,h) bone marrow (BM) on a FACSVantage DiVa cell sorter equipped with a krypton-ion laser emitting at an MLUV of 100 mW. Cells were incubated without (a,b) or with (c,d) fumitremorgin C before Hoechst 33342 labeling. The percentage of SP-positive cells is indicated for each Hoechst red versus blue cytogram (a–d). SP gates for these and all subsequent samples were drawn by using the fumitremorgin C inhibitor samples to set cutoffs. Simultaneous analysis of Sca-1 versus c-kit expression for unpurified and lineage-depleted samples is shown in e and f (with the percentage Sca-1-positive c-kit-positive cells indicated), and the Hoechst SP population for Sca-1-positive c-kit-positive fractions is shown in g and h.

tetraacetic acid, washed with cold HBSS/FBS/HEPES and counted before Hoechst 33342 labeling.

Hoechst 33342 Labeling for SP Discrimination

The above cell fractions, unseparated bone marrow, and A549 cells were then labeled with Hoechst 33342 by using the method described by Goodell et al. (1,3). Briefly, cells were resuspended at 10^7 cells/ml in HBSS with 2% FBS and 10 mM HEPES and prewarmed to 37°C. For some samples, bone marrow cells were preincubated with the ABCG2 inhibitor fumitremorgin C at 10 μ M for 15 min (9). Hoechst 33342 was then added at a final concentration of 5 μ g/ml, and the cells were incubated for 90 min at 37°C with periodic mixing. Cells were washed with cold HBSS/FBS/HEPES and simultaneously labeled with FITC-conjugated anti-Sca-1 and APC-conjugated anti-c-kit (BD Pharmingen). Cells were then washed with and resuspended in HBSS/FCS/HEPES and kept at 4°C until analysis (within 4 h).

Flow Cytometry

Cells were analyzed on one of two instruments: (a) a BD Bioscience FACSVantage DiVa jet-in-air sorter equipped with argon-ion, 488-nm, krypton-ion UV, and HeNe 633-nm lasers or (b) a BD Bioscience LSR II equipped with a solid-state, 488-nm, HeNe 633-nm and one of several violet or near-UV laser diode sources. Hoechst 33342-labeled cells were excited on the FACSVantage DiVa with

a Coherent I-302C krypton-ion laser emitting in multiline UV (MLUV) mode (351 and 357 nm) at 100 mW. Hoechst 33342-labeled cells were excited on the LSR II with one of the following lasers: (a) a Coherent VioFlame 408-nm violet laser diode emitting at 18 mW; (b) a Power Technology 401-nm violet laser diode emitting at 18 mW; or (c) a Power Technology 370-nm near-UV laser diode emitting at 8 mW. These diode sources were mounted on the BD LSR II as previously described (12,13), and a full laser mirror alignment and quality control check with fluorescent alignment microspheres was performed after all laser mountings. A variety of narrow bandpass filter combinations were used on the FACSVantage DiVa, with the Hoechst red and blue signals split with shortpass and longpass dichroics. Hoechst blue and red signals on the LSR II were detected with 450/50-nm and 650-nm longpass filters, respectively, split with a 595-nm longpass dichroic. Violet, near-UV, and UV laser alignments were quality controlled with 2- μ m yellow-green alignment beads (Polyscience, Warrington, PA); bead peak coefficients of variation (CVs) were less than 3.0 on the FACSVantage and less than 2.5 on the LSR II for all lasers before all experiments. Data was analyzed with WinMDI 2.8 (Joseph Trotter, BD Biosciences). SP gates were drawn by using the fumitremorgin C inhibitor samples to set cutoffs.

To express the relative detection resolution of the SP cells, data were expressed as a power function of Hoechst red and blue fluorescences calculated with Verity WinList

Violet diode 408 nm 25 mW

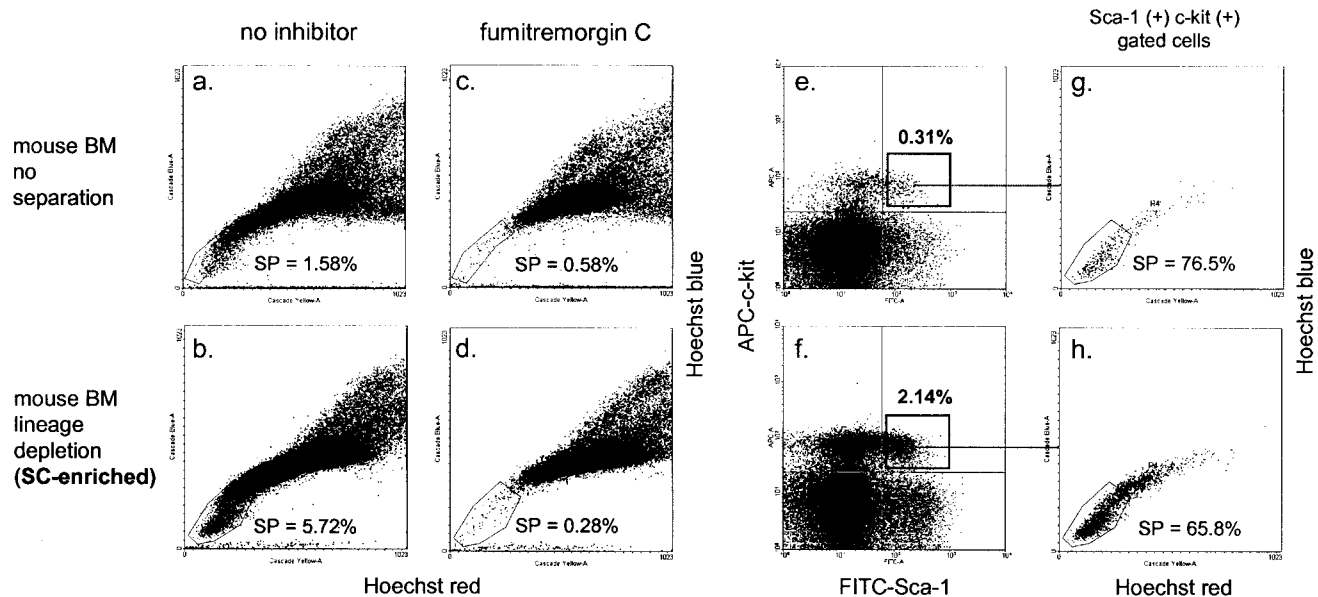


FIG. 3. Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted (b,d,f,h) bone marrow (BM) on a BD LSR II flow cytometer equipped with a violet laser diode emitting at 408 nm and 25 mW. Cells were incubated without (a,b) or with (c,d) fumitremorgin C before Hoechst 33342 labeling. The percentage of SP-positive cells is indicated for each Hoechst red versus blue cytogram (a–d). Simultaneous analysis of Sca-1 versus c-kit expression for unpurified and lineage-depleted samples is shown in e and f (with the percentage of Sca-1-positive c-kit-positive cells indicated), and the Hoechst SP population for Sca-1-positive c-kit-positive fractions is shown in g and h.

4.0 (Verity, Topsham, ME) and expressed as the CV of the resulting distribution.

RESULTS

Identification of the Hoechst 33342 SP in mouse bone marrow by flow cytometry traditionally has required high power levels of UV laser excitation. In this study we evaluated several newly available lower power diode laser sources for their ability to discriminate the SP in mouse bone marrow. Different commercial flow cytometers have different restrictions for the types of filters they can use; for example, the BD Bioscience LSR II employs only longpass dichroic filters to separate fluorescence signals. SP discrimination traditionally has required two narrow bandpass filters for the Hoechst blue and red signals, with signal separation by an intermediate shortpass filter. Fortunately, the filter requirements for SP detection are not strict; any Hoechst/4,6-diamidino-2-phenylindole blue filter and APC/Cy5 red filter combination can be used, and they can be split with a longpass or shortpass dichroic. This was confirmed in Figure 1, where Hoechst-labeled unseparated mouse bone marrow was analyzed with the traditional FACSVantage/UV gas laser system with several filter combinations shortpass and longpass splitters. The filter combination shown in Figure 1d most closely approximates the arrangement subsequently used on the BD LSR II: a relatively wide Hoechst blue filter (450/50), an open red longpass filter (650 LP), and a longpass dichroic. SP was equally well discriminated with all com-

binations, including this one. This flexibility in filter and dichroic selection suggests that the instrument type and filters/dichroics are not a critical factor for SP detection, and that we can carry out a valid comparison of several laser excitation sources on different instrument platforms.

Figure 2 shows SP analysis of unseparated (Fig. 2a, 2c, 2e, 2g) and lineage-depleted (Fig. 2b, 2d, 2f, 2h) mouse bone marrow cells untreated (Fig. 2a, 2b) or preincubated with the ABCG2 inhibitor fumitremorgin C (Fig. 2c, 2d). UV laser excitation provided excellent resolution of the SP cells, with good separation from the more differentiated bone marrow population. Simultaneous labeling of the bone marrow with FITC- and APC-conjugated antibodies against the murine stem cell markers Sca-1 and c-kit provided a confirmation of the identity of the SP. Sca-1 versus c-kit expression for both fractions is shown in Figure 2e and 2f; back-gating the Sca-1-positive c-kit-positive fraction gives a population highly enriched for SP cells and shows a relatively clear separation between SP and non-SP cells (Fig. 2g, 2h).

SP discrimination was then assessed with two violet laser diodes emitting at longer wavelengths than those generated by the UV gas laser. Violet laser diodes emitting at 408 and 401 nm were sequentially mounted on a BD Biosciences LSR II, with SP detection through 450/50 and 650 LP filters, split with a 595 LP dichroic. This filter combination, although different from that used for the combination shown in Figure 2, was validated on the

Violet diode 401 nm 18 mW

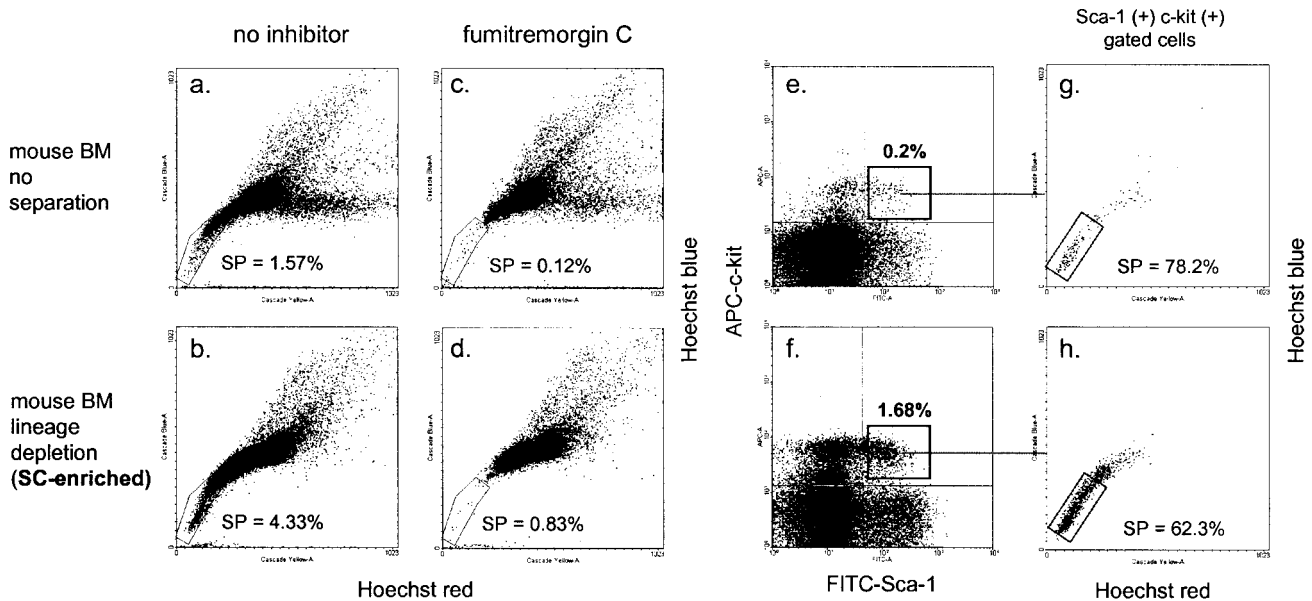


FIG. 4. Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted (b,d,f,h) bone marrow (BM) on a BD LSR II flow cytometer equipped with a violet laser diode emitting at 401 nm and 18 mW. Cells were incubated without (a,b) or with (c,d) fumitremorgin C before Hoechst 33342 labeling. The percentage of SP-positive cells is indicated for each Hoechst red versus blue cytogram (a–d). Simultaneous analysis of Sca-1 versus c-kit expression for unpurified and lineage-depleted samples is shown in e and f (with percentage Sca-1-positive c-kit-positive cells indicated), and the Hoechst SP population for Sca-1-positive c-kit-positive fractions is shown in g and h.

FACSVantage, as shown in Figure 1. Figures 3 and 4 show the results for the 408- and 401-nm lasers, respectively. An SP was visible in Figures 3a and 3b (for 408 nm) and 4a and 4b (for 401 nm). The discrimination between SP and non-SP cells approaching the G1 peak was approximately the same as that for the MLUV analysis, but its resolution as defined by the population “sharpness” of the SP cells was poorer than that observed for the UV laser. Simultaneous labeling for Sca-1 and c-kit with subsequent back-gating into a Hoechst cytogram demonstrated that the SP did indeed conform to the same functional characteristics as that discriminated by the UV laser; however, a significant amount of population peak resolution “sharpness” was lost with violet excitation. This loss of sensitivity was likely not related to the power level of the laser; excitation by krypton-ion 407-nm violet gas lasers at power levels up to 100 mW similarly gave a poorly resolved SP population, and low-power UV sources such as HeCad lasers have been shown to successfully resolve SP at power levels below 10 mW (data not shown; Teresa Hawley, personal communication).

Violet excitation does appear to be adequate, however, for cell lines expressing high levels of ABCG2 activity. Figure 5 shows a comparison between Hoechst-labeled mouse bone marrow cells (Fig. 5a–d) and A549 lung carcinoma cells (Fig. 5e–h) with krypton-ion UV (Fig. 5a, 5b, 5e, 5g) or violet laser diode at 408-nm (Fig. 5b, 5d, 5g, 5h) excitation. Mouse bone marrow run in parallel showed the expected loss of resolution, but A549 cells

showed a similar SP pattern with either excitation source. Cell lines or types with high levels of ABCG2 pump activity therefore may be amenable to SP analysis with violet laser sources.

As illustrated above, however, near-UV or true UV excitation appeared to be a requirement for acceptable SP resolution based on the “sharpness” of the population. Recent developments in laser diode technology have resulted in practical gallium nitride diodes emitting in the near-UV range. A prototype Power Technology near-UV laser diode emitting 8 mW at 370 nm was therefore mounted on the LSR II and tested for its ability to resolve SP. The results are shown in Figure 6, where unseparated or lineage-depleted bone marrow was analyzed with a krypton-ion UV (Fig. 6a–d) or the near-UV 370-nm (Fig. 6e–h) sources. Resolution of the SP with the near-UV laser diode was excellent, despite its relatively low power level. These results demonstrated that the near-UV diodes have reached the wavelength and power threshold for adequate SP discrimination when analysis is carried out on a cuvette-based instrument.

The resolution at the cutoff point between SP and non-SP cells was relatively similar for all laser sources; the “sharpness” of the SP population on Hoechst red and blue axes, however, was much better for the near-UV and UV sources. To quantify this somewhat subjective appearance characteristic, SP data were expressed as a power function of Hoechst red and blue values, with the resulting distribution reflecting the peak and skew of both param-

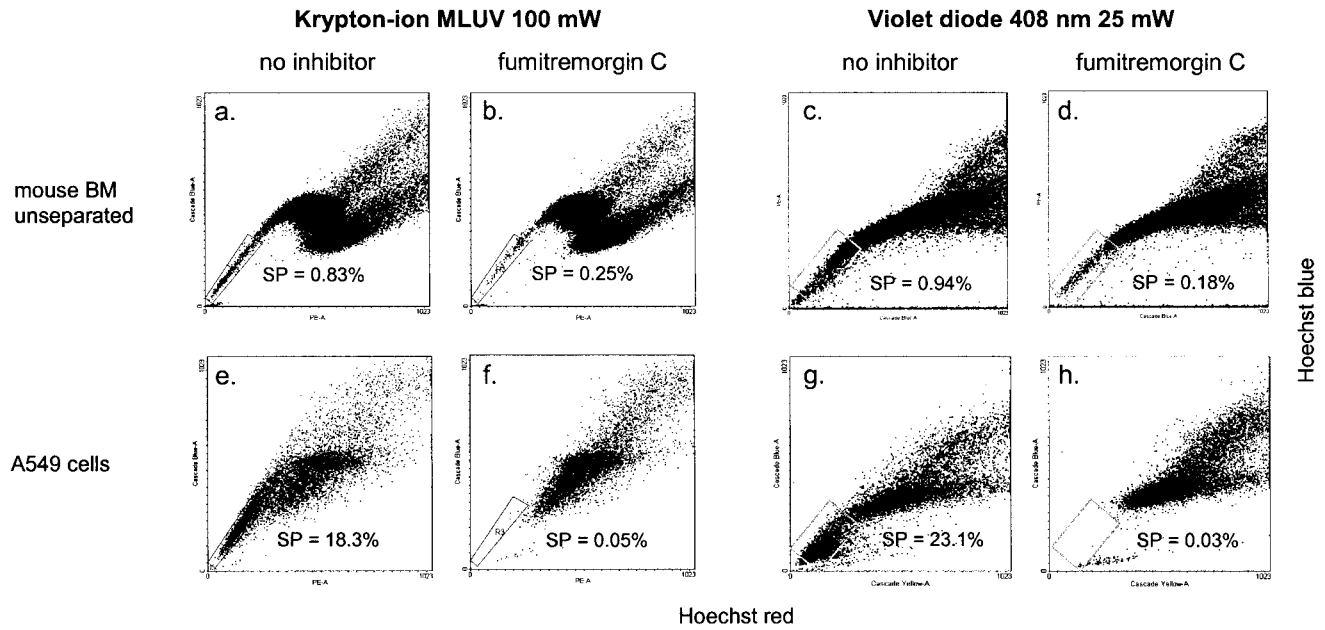


FIG. 5. Hoechst SP analysis of unpurified bone marrow (a–d) or A549 lung carcinoma cells (e–h) on a FACSVantage DiVa cell sorter equipped with a krypton-ion laser emitting at an MLUV of 100 mW (a,b,e,f) or a BD LSR II flow cytometer equipped with a violet laser diode emitting at 408 nm and 25 mW (c,d,g,h). Cells were incubated without (a,c,e,g) or with (b,d,f,h) fumitremorgin C before Hoechst 33342 labeling. The percentage of SP-positive cells is indicated for each Hoechst red versus blue cytogram.

eters. The resulting distributions and CVs for violet diode, krypton-ion MLUV, and near-UV diode sources is shown in Figure 7. Both violet diode sources produced rela-

tively broad CVs; the UV and near UV sources produced much lower values that were comparable to one another.

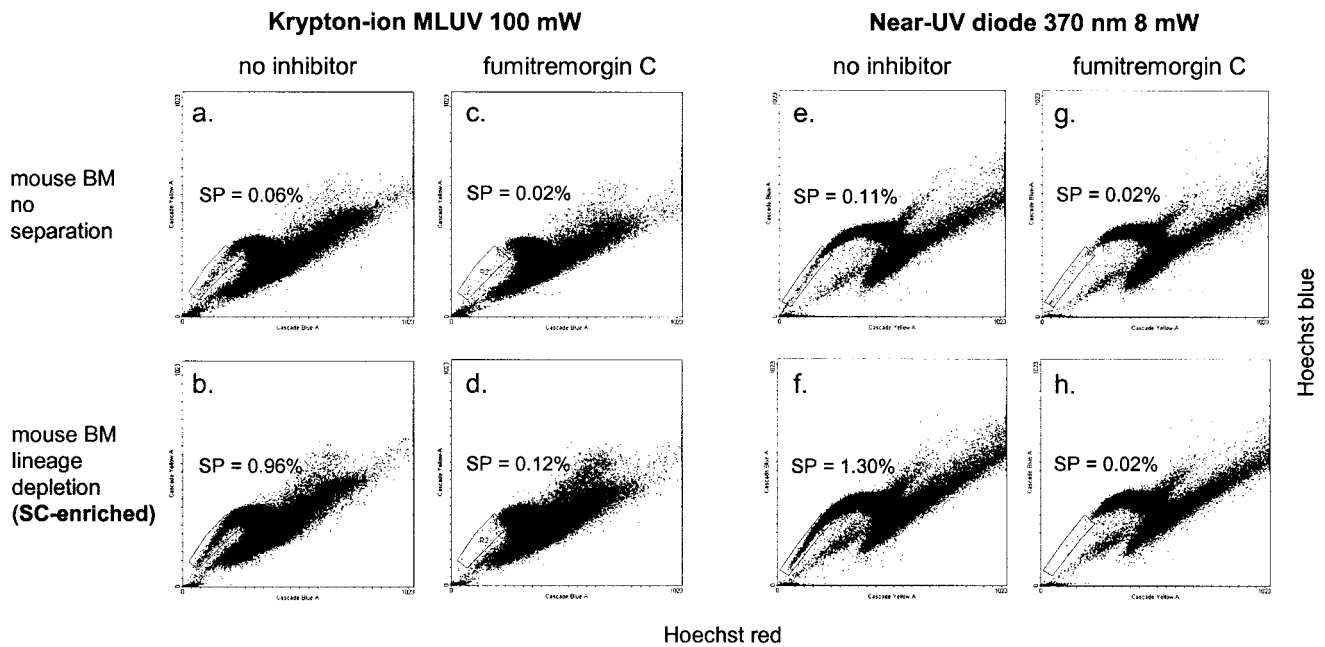


FIG. 6. Hoechst SP analysis of unpurified (a,c,e,g) and lineage-depleted (b,d,f,h) bone marrow on a FACSVantage DiVa cell sorter equipped with a krypton-ion laser emitting at an MLUV of 100 mW (a–d) or a BD LSR II flow cytometer equipped with a near-UV laser diode emitting at 370 nm and 8 mW (e–h). Cells were incubated without (a,b,e,f) or with (c,d,g,h) fumitremorgin C before Hoechst 33342 labeling. The percentage of SP-positive cells is indicated for each Hoechst red versus blue cytogram.

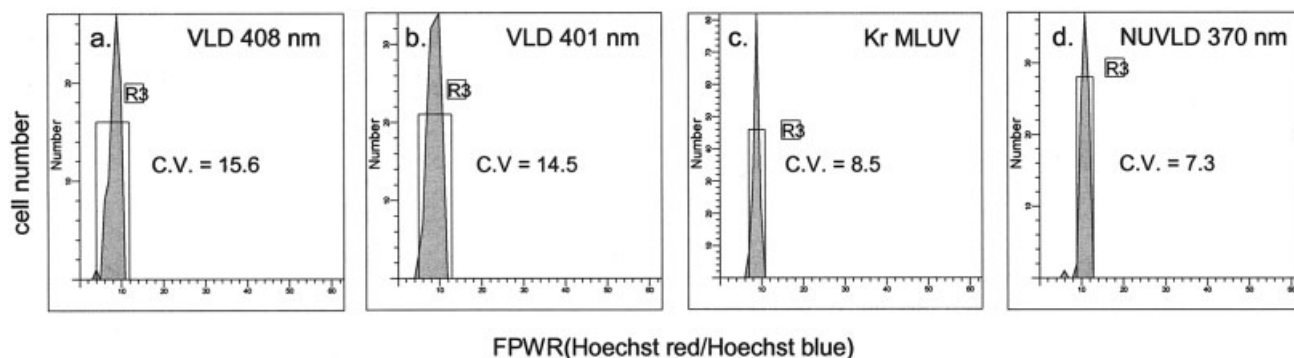


FIG. 7. Hoechst SP analysis of lineage-depleted bone marrow by using the violet diode at 408 nm and 25 mW (a), violet diode at 401 nm and 18 mW (b), MLUV of 100 mW (c), or near-UV diode at 370 nm and 8 mW (d). Data are expressed as a power function of Hoechst red and blue fluorescence, with the CV calculated from the resulting distribution (indicated on the figure). SP cells were gated by using the appropriate funitremorgin C control for setting the population cutoff.

DISCUSSION

These results demonstrated that violet laser diodes, although providing SP discrimination at the level of SP and non-SP separation and subsequent enrichment by Sca-1/c-kit back-gating, do not convey the sharp SP peak resolution observed with traditional UV sources. In contrast, near-UV diode sources do appear to provide the same level of sensitivity as far more powerful UV gas lasers on traditional jet-in-air flow systems, based on the overall appearance of the population and on the calculation of multiparameter peak CV. The inability of violet laser diodes to provide adequate or appropriate excitation for SP is disappointing (given the recent proliferation of these lasers in commercial instruments) but not surprising; the excitation spectra for Hoechst indicates only 2–8% excitation efficiency in the 401- to 408-nm range, and even conventional krypton-ion violet excitation at higher power levels produces poor SP discrimination. Excitation at 401 nm produced marginally better SP appearance than excitation at 408 nm, however, suggesting that a further decrease in excitation wavelength would improve SP discrimination. Excitation at 370 nm gave SP discrimination at least as good as that achieved with much more powerful gas lasers. These results not only confirm the utility of near-UV lasers for SP analysis (with slightly longer wavelengths than gas versions) but also the ability of relatively low-power diode sources to detect SP cells when used with sensitive cuvette-based instruments. Because small, inexpensive diode lasers can be incorporated more easily into a larger variety of flow cytometers than large gas sources, these findings have the potential of making Hoechst SP analysis far more accessible to biomedical researchers.

Although these results demonstrate that Hoechst SP analysis can be carried out on smaller, bench-top flow cytometers equipped with near-UV laser diodes, the larger cell sorters with gas lasers are still required for physical sorting of SP cells. The jet-in-air sample handling of these instrument results in a significant decrease in light collection efficiency and ultimately of instrument sensitivity.

Whereas low-power violet laser diodes have been found to work reasonably well on these instruments, near-UV sources still do not possess the power levels necessary to achieve good Hoechst SP discrimination on these instrument types. The next generation of cell sorters, however, incorporate closed cuvette cell analysis and subsequent jet-in-air sorting; these instruments (which include the BD Bioscience FACS Aria, among others) have far more efficient light collection optics, allowing the integration of lower-power solid-state laser sources. Violet laser diodes are already a standard fixture on such instrumentation. Minimal engineering modifications should be necessary to incorporate near-UV laser diode sources into this instrumentation, making sensitive Hoechst SP analysis and sorting possible with these novel laser sources.

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LITERATURE CITED

- Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;183:1797–1806.
- Goodell MA, Rosenzweig M, Kim H, Marks DF, De Maria M, Paradis G, Grupp S, Seiff CA, Mulligan RC, Johnson RP. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997;3:1337–1345.
- Goodell MA. Stem cell identification and sorting using the Hoechst 33342 side population (SP). In: Robinson JP, Darzynkiewicz Z, Dean PN, Hibbs AR, Orfao A, Rabinovitch PS, Wheelless LL, editors. *Current protocols in cytometry*. New York: John Wiley & Sons; 2001. p 9.18.1–9.18–21.
- Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates SE. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). *J Cell Sci* 2000;113:2011–2021.

5. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, Lagutina I, Grosveld GC, Osawa M, Nakauchi H, Sorrentino BP. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.* 2001;7:1028-1034.
6. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002; 99:507-512.
7. Uchida N, Leung FY, Eaves CJ. Liver and marrow of adult mdr-1a/1b(-/-) mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells. *Exp Hematol.* 2002;30: 862-869.
8. Kim M, Turnquist H, Jackson J, Sgagias M, Yan Y, Gong M, Dean M, Sharp JG, Cowan K. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. *Clin Cancer Res* 2002;8: 22-28.
9. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, Schellens JH, Koomen GJ, Schinkel AH. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* 2002;1:417-425.
10. Doornbos RMP, De Grooth BG, Kraan YM, Van Der Poel CJ, Greve J. Visible diode lasers can be used for flow cytometric immunofluorescence and DNA analysis. *Cytometry* 1994;15:267-271.
11. Nakamura S, Fasol G. The blue laser diode. GaN based light emitters and lasers. Berlin: Springer-Verlag; 1997.
12. Shapiro HM, Perlmutter NG. Violet laser diodes as light sources for cytometry. *Cytometry* 2001;44:133-136.
13. Telford WG, Hawley TS, Hawley RG. Analysis of violet-excited fluorochromes by flow cytometry using a violet laser diode. *Cytometry* 2002;(suppl 11):123.
14. Telford WG, Hawley TS, Hawley RG. Analysis of violet-excited fluorochromes by flow cytometry using a violet laser diode. *Cytometry* 2003;54A:48-55.
15. Telford WG. Small lasers in flow cytometry. In: *Flow cytometry*. London: Humana Press. Forthcoming.